



King's Research Portal

DOI:

[10.1021/acs.molpharmaceut.6b00448](https://doi.org/10.1021/acs.molpharmaceut.6b00448)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Patel, A., Rudman, S. D., Brown, M., Hider, R. C., Jones, S. A., & Page, C. P. (2016). Using Salt Counterions to Modify 2Agonist Behavior in Vivo. *Molecular Pharmaceutics*, 13(10), 3439-3448.
<https://doi.org/10.1021/acs.molpharmaceut.6b00448>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Using salt counter-ions to modify β_2 -agonist behaviour *in vivo*

Aateka Patel^{*†‡}, Sandra D. Keir^{†‡}, Marc B. Brown^{§‡}, Robert Hider[‡], Stuart A. Jones[‡], Clive P. Page^{†‡}

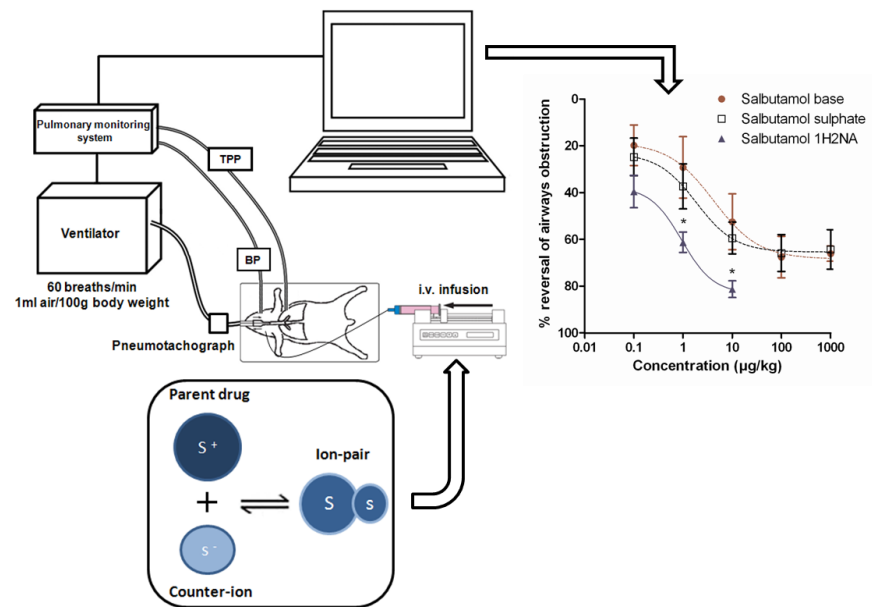
[†]Sackler Institute of Pulmonary Pharmacology, Faculty of Life Sciences & Medicine, King's College London, 150 Stamford Street, London SE1 9NH London, UK

[‡]Institute of Pharmaceutical Science, Faculty of Life Sciences & Medicine, King's College London, 150 Stamford Street, London SE1 9NH, UK

[§]MedPharm Ltd, Unit 1 and 3, Chancellor Ct, 50 Occam Rd, Surrey Science Park, Guildford, Surrey GU2 7AB, UK

[‡]School of Pharmacy, University of Hertfordshire, College Lane, Hatfield, Herts, AL10 9AB, UK

Table of Contents/Abstract Graphic



ABSTRACT

There are a paucity of data describing the impact of salt counter-ions on the biological performance of inhaled medicines *in vivo*. The aim of this study was to determine if the co-administration of salt counter-ions influenced the tissue permeability and the airway smooth muscle relaxation potential of salbutamol, formoterol and salmeterol. The results demonstrated that only salbutamol, when formulated with an excess of the 1-hydroxy-2-naphthoate (1H2NA) counter-ion, exhibited a superior bronchodilator effect ($p < 0.05$) compared to salbutamol base. The counter-ions aspartate, maleate, fumarate and 1H2NA had no effect on the ability of formoterol or salmeterol to reduce airway resistance *in vivo*. Studies using guinea pig tracheal sections showed that the salbutamol:1H2NA combination resulted in a significantly faster ($p < 0.05$) rate of tissue transport compared to salbutamol base. Furthermore, when the relaxant activity of salbutamol was assessed *in vitro* using electrically stimulated, superfused preparations of guinea pig trachea, the inhibition of contraction by salbutamol in the presence of 1H2NA was greater than with salbutamol base (a total inhibition of 94.13 %, $p < 0.05$). The reason for the modification of salbutamol's behavior upon administration with 1H2NA was assigned to ion-pair formation, which was identified using infrared spectroscopy. Ion-pair formation is known to modify a drug's physicochemical properties and the data from this study suggested that the choice of counter-ion in inhaled pharmaceutical salts should be considered carefully as it has the potential to alter drug action *in vivo*.

KEYWORDS: salbutamol; ion-pair; airways; counter-ion; pharmaceutical salt; 1H2NA

ABBREVIATIONS: 1H2NA, 1-hydroxy-2-naphthoate; β_2 -AR, β_2 -adrenoceptor; COPD, chronic obstructive pulmonary disorder; i.m., intra-muscular; i.v., intra-venous; SEM, standard error of the mean; RPHPLC, reverse phase high performance liquid chromatography; SD, standard deviation; K-H, Krebs-Henseleit; EFS, electrical field stimulation; ANOVA, analysis of variance; SAL, salbutamol base; FT-IR, Fourier transform infra red; P_{app} , apparent permeability; ASM, airway smooth muscle; OCTs, organic cation transporters.

1. Introduction

A pharmaceutical salt is particularly susceptible to the composition of bodily fluids into which it dissolves because there is the potential that the drug can partake in a number of different non-covalent physical interactions. For example, if the pharmaceutical salt dissolves into a fluid that allows the drug to ionise, ion-pairs can be formed in solution.¹ Ion-pairs usually arise from the drug interacting with co-administered salt counter-ions and they can modify a drug's physicochemical behaviour.²

Not every pharmaceutical salt will be susceptible to ion-pair formation upon dissolution in bodily fluids.³ The formation of an ion-pair complex between the ionised drug and co-administered ionised counter-ion is dependent upon the physicochemical properties of the drug, the physicochemical properties of the ion-pair, the affinity of the two molecules and the route of administration.¹ For example, when administering a pharmaceutical salt orally the drug is exposed to a relatively large volume of liquid and hence, if a liable, non-covalent bond is formed between the drug and the counter-ion it will most probably be broken when faced by rapid dilution and competition from charged species in the gut fluids, unless the ion-pair binding is very strong. Such a scenario has led to the regulatory approval of several salt forms of the same

drug for a single therapeutic indication when dosed orally because the different salt forms appear to be bio-equivalent.⁴ However, this does not mean that all salts are bioequivalent. The biological consequences of delivering therapeutic agents as pharmaceutical salts via routes which do not expose the molecules to such a large volume of fluid, e.g. the skin and the airways, may be more significant compared to oral administration.

Previous literature has suggested that changes in drug transmembrane permeation as a result of ion-pair formation has a major influence on the ability of salt counter-ions to modify the parent drugs behaviour.⁵⁻⁷ In addition, data generated *in vitro* has suggested that ion-pairs can remain intact upon crossing biological barriers⁸ and therefore it may be possible that a parent drug's behaviour may be influenced by a salt counter-ion, even after absorption into the body. However, there appears to be very limited information about the consequence of ion-pair formation *in vivo*.⁹ This is surprising given the number of pharmaceutical salts that are used clinically and it suggests that further research into the manner of how co-administered counter-ions influence the biological behaviour of pharmaceutical salts is required.

The primary aim of this study was to investigate the potential of a co-administered counter-ion to alter the biological behaviour of a drug, which forms a pharmaceutical salt, *in vivo*. The inhaled route was chosen for this study due to the low volume of liquid in which the drug and counter-ion dissolves and the fact that the ability of an ion-pair to alter the physicochemical properties of an inhaled drug has already been established.¹⁰ The β_2 -adrenoceptor (AR) agonists, salbutamol, salmeterol and formoterol were selected as the drug molecules in this work because they are all commercially available as pharmaceutical salts and are widely used clinically for the treatment of asthma and chronic obstructive pulmonary disease (COPD).¹¹ In addition, salbutamol has already been studied *in vitro* with a number of counter-

ions including, sulphate,^{10,12} adipate, stearate,¹⁰ succinate,¹² acetate monomethanolate,¹³ and hydrochloride,¹⁴ and this provided vital information upon which to base the *in vivo* work. As the physical and chemical properties of the salbutamol salts have already been characterized^{10,12,15} this study initially focused on the pharmacodynamic consequences of drug-counter-ion co-administration. However, to better understand the drug-counter-ion mixtures that elicited changes in pharmacodynamics responses, an attempt was made to characterise the strength of the drug counter-ion interactions, to elucidate the effects of the counter-ions upon tissue permeation, and understand the influence of the counter-ions upon the drug's pharmacological action. The counter-ions aspartate, maleate, fumarate and 1H2NA were selected to represent a series of compounds that were used clinically, displayed different physicochemical properties, and had the potential to form ion-pairs with the β_2 -AR agonists. The drug-counter-ion combinations used in the study were based upon the molecules compatibility in the solution state at the concentrations that were used in the work. As it was anticipated that the interaction affinity between the drug and counter-ions would be relatively weak the therapeutic agents were administered in all the studies with an excess of counter-ions to maximise the interactions between the β_2 -AR agonist and the counter-ions. Whilst it was accepted that this does not necessarily mimic the use of a pharmaceutical salt *in vivo*, as they are typically presented to the body with lower counter-ion concentrations, the experiments were designed in this manner to continually present the test agents to different *in vitro* and *in vivo* systems in a similar state, i.e., if they formed ion-pairs a drug that was fully bound to the counter-ion, even within the fluids used in assays varied. This was an essential experimental design trait of this work that allowed the study data to be directly compared across the test systems. In addition, to mimic the inhaled administration of 'pharmaceutical salts', which typically are produced as powders, a production method for each

drug-counter-ion combination would be required to produce the lowest energy salt form in the solid-state. This would be a considerable piece of work and the reporting of the physical data, which would be needed to support the use of these compounds, would detract from the *in vivo* aspect of the study which was our focus. Therefore simple solutions of the drugs and the counter-ions were used in all the test systems reported in this work.

2. Materials and Methods

2.1. Materials and reagents

Salbutamol base (BN. H80619) was a kind gift from Cipla Ltd., Mumbai, India. Salbutamol sulphate (BN. B027798) was obtained from GlaxoSmithKline Research and Development, Ware, UK. Salmeterol xinafoate (BN. SX-0081010) was purchased from Vamsi Labs, India. Salmeterol base and formoterol base were synthesised by Tocris Cookson Ltd., Avonmouth, Bristol, UK. Formoterol fumarate dihydrate (BN. 27000M0 A0031421) was obtained from Arena Pharmaceuticals, Buckingham, UK. Phosphate Buffered Saline (PBS) tablets (0.15 M, pH 7.3) were purchased from Oxoid, Basingstoke, UK. WhatmanTM nylon filters (pore size 0.2 μ m, diameter 47 mm) were purchased from Fisher Scientific Ltd., Loughborough, UK. Methanol (Fisher Scientific Ltd, UK) and ammonium acetate (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) were of high performance liquid chromatography (HPLC) grade. Indomethacin, sodium hydroxide, hydrochloric acid, 1-hydroxy-2-naphthoate (1H2NA), bombesin acetate, maleic acid, L-aspartic acid, sodium chloride, potassium chloride, calcium chloride dihydrate, magnesium sulphate, sodium bicarbonate, potassium dihydrogen phosphate,

and D-glucose were all purchased from Sigma-Aldrich Company Ltd., Poole, Dorset, UK and were of analytical grade.

2.2. *Animals*

Experiments were conducted under a project licence issued by the United Kingdom Home Office in accordance with the United Kingdom Animal Scientific Procedures Act, 1986. Protocols were approved by the Local Ethical Review Committee of King's College London. All *in vivo* experiments were performed with male Dunkin-Hartley guinea pigs (250 - 500 g, Charles River). Guinea pigs were housed in animal holding facilities maintained at 18 - 19 °C with a standard 12 hour light-dark cycle. A maximum of six guinea pigs were housed per cage, containing sawdust and hay with cardboard tunnels in order to provide environmental enrichment. Animals were fed *ad libitum* on a FD1 guinea pig diet (Special Diets Services, Witham, Essex, UK) with free access to water.

2.3. *Lung function studies*

Male Dunkin-Hartley guinea pigs (250 – 500 g) were anaesthetised with urethane (25 % w/v, 7 ml kg⁻¹, i.p.) and a neuromuscular blocker was also administered to induce skeletal muscle paralysis (succinylcholine chloride, 1 mg kg⁻¹, i.m). The trachea, carotid artery and both jugular veins were cannulated for measurement of airway obstruction, systemic blood pressure, and for i.v. administration of test substances, respectively. An i.v. infusion was used to dose the ion-pairs, as this minimised the potential of non equivalent doses reaching the tissue as a consequence of differences in deposition and absorption via the inhaled route. The tracheal cannula was attached to a pneumotachograph that was in turn connected to a pressure transducer

(± 2 cm H₂O, model MP-45-14-871, Validyne Engineering). Changes in airflow were measured using a lung function recording system (version 9.2; Mumed Systems, London, UK) and displayed in real time on a personal computer. The flow signal was integrated to give a measure of tidal volume. A cannula was inserted into the thoracic cavity between the third and fifth ribs and connected to the negative side of the pressure transducer (± 20 cm H₂O, Validyne). The positive side of the pressure transducer was connected to the side of the pneumotachograph proximal to the animal to obtain a measure of transpulmonary pressure (difference between mouth and thoracic pressure). The lung function parameter, total airway resistance (R_L ; cm of water per L per sec), was derived from each measure of flow, tidal volume, and transpulmonary pressure by the method of integration. Animals were allowed to acclimatise for 10 min prior to the start of the study.

For bronchodilator experiments, bronchoconstriction was induced by means of continuous i.v. administration of bombesin (2 - 4 $\mu\text{g kg}^{-1}$) which elicited sustained submaximal increases in total airway resistance, as described elsewhere.¹⁶ After stable bronchoconstriction had been obtained, salbutamol, formoterol or salmeterol base, alone or in the presence of the counter-ions, were cumulatively (0.1, 1, 10, 100 or 1000 $\mu\text{g kg}^{-1}$) administered through a separate cannula to reverse the bombesin-induced bronchoconstriction. Salbutamol was formulated with sulphate as it was supplied (2:1 ratio) with a salbutamol concentration of 0.1, 1, 10, 100 or 1000 mg kg^{-1} , and 1H2NA at a molar ratio of 1:10. Formoterol was formulated with maleate or aspartate at a ratio of 1:10, formoterol fumarate was used as supplied with a formoterol concentration of 0.1, 1, 10, 100 or 1000 mg kg^{-1} . Salmeterol was formulated with maleate or aspartate at a ratio of 1:10 and a salmeterol concentration of 0.1, 1, 10, 100 or 1000 mg kg^{-1} . The dose was increased when the effect of the previous dose had reached a plateau. Bronchodilation was calculated as the

percentage reversal of the increase in total lung resistance induced by bombesin. The bronchodilator potency was calculated as the effective dose of salbutamol that reversed bombesin-induced bronchoconstriction by 50%. Data were expressed as arithmetic means \pm standard error of the mean (SEM).

2.4. Salbutamol counter-ion interaction studies

For the analysis of salbutamol in the presence of the hydrophobic counter-ion 1H2NA a UV/Vis spectrophotometer (Perkin Elmer Ltd., Beaconsfield, UK) was used. Standard stock solutions of salbutamol base and 1H2NA were prepared in de-ionised water (pH 6.5) measured using a pH meter and adjusted using 0.1 M sodium hydroxide and/or hydrochloric acid. Solutions of salbutamol base and 1H2NA were mixed to achieve a concentration range of 0.005-0.84 mM for 1H2NA and a constant salbutamol base concentration of 0.42 mM, made up to volume using de-ionised water. The final pH of the samples were checked and adjusted to pH 6.5. Samples were scanned between 190 and 400 nm using a UV Quartz cuvette (path length 10 mm). A background reading of a 'blank' solution was preceded by three replicate readings of each sample. The spectral data was converted into an Excel file for analysis.

For the infra-red (IR) studies solutions of salbutamol base and salbutamol in the presence of sulphate were prepared in deuterated water, and deuterated methanol (80:20). Solutions of salbutamol base and salbutamol in the presence of 1H2NA were prepared in a 70:30 ratio of deuterated methanol to deuterated water. All samples were adjusted to pH 6.5 using 0.1 M sodium hydroxide and/or hydrochloric acid, and checked again at the end of the study. Spectra's were obtained with the aid of a universal Omni-Cell system (Specac Ltd., Orpington, Kent, UK)

for the analysis of liquids equipped with calcium fluoride (CaF_2) windows (Mylar spacer at 0.025 mm path length). A double solvent subtraction procedure was employed for all spectra's. Samples were scanned on average 32 times, with a resolution of 4 cm^{-1} over the 550-4000 cm^{-1} range. Data was recorded using a Spectrum One spectrometer and spectral analysis was performed with Spectrum version 5.3.1 software (Perkin Elmer Ltd., Beaconsfield, UK).

2.5. Lung tracheal transport studies

Transport experiments were carried out in unjacketed, individually calibrated, upright Franz diffusion cells (MedPharm Ltd., UK). Guinea pigs were killed by a blow to the head, followed by exsanguination; the trachea was excised and placed in cold physiological saline (0.9 % sodium chloride, pH 5.5). The adherent connective tissue was dissected away and the lumen gently flushed with saline. The trachea was dissected into rings consisting of 5 - 6 cartilage bands and mounted between the donor and receiver chambers, which were sealed together using parafilm. Small magnetic bars were inserted into each of the receiver compartments to ensure adequate mixing. The receiver compartments were filled with physiological saline (0.9 % sodium chloride, pH adjusted to 7.4) and each cell was checked by inversion for leaks. The assembled Franz cells were placed in a 37°C water bath and allowed to equilibrate for 60 min prior to adding 300 μL of the donor solution (salbutamol base, salbutamol sulphate (commercial salt) or salbutamol in the presence of 1H2NA). The salbutamol base concentration was fixed at 0.00178 M for all test systems. An excess of 1H2NA was presented at a concentration of 17.8×10^{-3} M to mimic the in vivo studies. All samples were made up to volume using de-ionised water (pH 7.4) and the final pH adjusted to 7.4 using 0.1 M sodium hydroxide or hydrochloric acid. Samples

(0.2 mL) were taken for reverse phase high performance liquid chromatography (RPHPLC) analysis (method I or II detailed below) at 0 (prior to addition of the donor solution), 15, 30, 45, 60, 120, 180, 240, 300, 360, 1320, 1380 and 1440 min after the donor solution was added to the cells. Following sampling each receiver cell compartment was replaced with fresh pre-warmed physiological saline (0.9 % sodium chloride, pH adjusted to 7.4) of equal volume. The cumulative amount of drug (μg) transported through the trachea per unit area (cm^2) was corrected for sample removal and plotted against time (min). Steady-state flux was calculated using the line of best fit over a minimum of five time points with a linearity of $R^2 \geq 0.97$ and displayed as mean \pm standard deviation (SD). Where appropriate a two-tailed, unpaired, Student's *t*-test was used and statistically analysed at the 95% confidence level.

2.6. Reverse phase high performance liquid chromatography (RPHPLC)

Method I - Separation of salbutamol base and salbutamol sulphate was achieved using a SphereClone ODS2 (250 x 4.6 mm, particle size: 5 μm) column (Phenomenex® Ltd., Cheshire, UK) maintained at 50°C using a thermostatted column compartment, TCC-100 (Dionex Corporation, Sunnyvale, CA, USA). The mobile phase was a mixture of aqueous ammonium acetate solution - 0.1% and methanol (80:20) at pH 4.5, filtered through a 0.2 μm nylon membrane and degassed by sonication. The mobile phase flow rate was 1.0 mL min⁻¹, the injection volume was 20 μL and detection was set at 272 nm. Quantitative determination of the active substance was performed using a RPHPLC system consisting of a Dionex P680 pump, a Dionex PDA-100 photodiode array detector and a Dionex ASI-100 automated sampler injector with Chromeleon Client version 6.60 for analysis (Dionex Corporation, Sunnyvale, CA, USA).

Method II - Separation of salbutamol base and salbutamol in the presence of 1H2NA was achieved using a SphereClone ODS2 column, maintained at 50°C using a thermostatted column block heater TCC-100. The mobile phase was a mixture of aqueous ammonium acetate solution - 0.1% and methanol (80:20) at pH 6.0, filtered using a through a 0.2 µM nylon membrane and degassed by sonication. The stationary phase was used with a 1.2 mL min⁻¹ flow rate and a 20 µL injection volume. The compounds were analysed at a wavelength of 276 nm.

Both RPHPLC methods were shown to be fit for purpose in terms of precision ($\leq 2\%$), accuracy ($\geq 99\%$), linearity ($R^2 > 0.9999$) and sensitivity (limit of detection $< 0.53 \mu\text{g mL}^{-1}$; limit of quantification $< 1.78 \mu\text{g mL}^{-1}$). A two-tailed, unpaired, Student's *t*-test determined no significant ($p > 0.05$) differences on average retention times between salbutamol base assayed alone or in the presence of counter-ions.

2.7. In vitro contractility of isolated guinea pig trachea

Superfusion of guinea pig tracheal rings was performed according to a previously described method.^{17,18} Guinea pigs were sacrificed by cervical dislocation, and the trachea were excised and cut into rings. Each tracheal ring (3 - 5 cartilage bands) was opened by sectioning the ring opposite the smooth muscle and sectioned to form strips. A cotton thread was attached to the cartilage at one end of the strip for attachment to the tension gauge, and a cotton loop to the other end for anchoring the tissue to a fixed stainless steel hook. The tissue was then suspended between two platinum electrodes under 1-g tension and superfused at a rate of 3 mL min⁻¹ (Watson-Marlow Sci-Q 323S peristaltic pump and a 318MC eight roller five channel micro pump head, Cornwall, U.K.) through a jacketed, glass heat exchanger with Krebs-Henseleit (K-

H) solution (37°C, 5% CO₂, 95% O₂) containing the cyclooxygenase inhibitor, indomethacin (5 µM) to inhibit the release of endogenous prostaglandins and reduce spontaneous tone of the airway smooth muscle.

K-H solution (pH 7.2 – 7.4) was composed of: 118 mM sodium chloride, 4.7 mM potassium chloride, 2.5 mM calcium chloride dihydrate, 1.2 mM magnesium sulphate, 25 mM sodium bicarbonate, 1.2 mM potassium dihydrogen phosphate, and 11.1 mM D-glucose made up to volume in de-ionised water.

The tracheal preparations were equilibrated for 40 min before commencement of electrical field stimulation (EFS) delivered as 10 s trains of square wave pulses at 3 HZ, 0.1 ms duration and 30 v generated every 100 s by means of a physiological square wave stimulator (Digitimer MultistimSystem-D330, Digitimer Ltd, Herts, U.K.). Changes in tension detected by the tension gauge were recorded using a Powerlab/8sp (AD Instruments, Castle Hill, Australia). Ten minutes after the initiation of airway smooth muscle stimulation, the trachea was superfused with K-H solution containing the drug of interest by slow infusion. The drug was delivered at a rate of 0.275 mL min⁻¹ using a syringe pump (Original-Perfusor-Spritze, B. Braun Medical, Melsungen, Germany) to which a butterfly-25G short winged infusion tube (Abbott, Sligo, Republic of Ireland) was attached for 20 min, at which point superfusion with drug was terminated. The tissues were then superfused with drug free K-H solution and tension was monitored for a further 6 h. The maximum-minimum (g) peak height for the period of contraction was calculated offline by using LabChart 7 Reader software (AD Instruments, Hastings, UK).

All EFS data are expressed as percentage inhibition of the contractile response obtained prior to the administration of the drug and displayed as mean ± standard error of the mean

(SEM). Onset time (OT₅₀ and OT₇₅) was expressed as the time taken from administration of the drug to achieve 50% or 75% control (pre-drug) value (minutes) and was determined by interpolation from the plot of percentage against time to attain 50% or 75% inhibition of the response. Likewise, the recovery (or offset) time (RT₅₀ and RT₇₅) was expressed as the time taken from stopping the administration of the drug to achieve 50% or 75% recovery of the contractile response (pre-drug) value (minutes). It was determined by interpolation from a plot of percentage response (% control) against time to attainment of 50% or 75% recovery from that response.

Data was analysed using a Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) with post-hoc Dunn's Multiple Comparison test. The final concentration of salbutamol base on the tissue remained constant at 1 μ M or 10 μ M. The hydrophobic counter-ion, 1H2NA, was added to salbutamol base (1 μ M) in excess of 10 fold (10 μ M). Salbutamol sulphate was at a total concentration of 1 μ M. All samples were made up to volume in de-ionised water and adjusted to pH 6.5 or 7.4 with 1 M sodium hydroxide and/or hydrochloric acid.

2.8. Data and statistical analysis

All data analysis was performed using Graphpad Prism (Prism, version 5; GraphPad Software Inc., San Diego, CA). Data was analysed for normality using the Anderson-Darling test. Where appropriate a two-tailed, unpaired, Student's *t*-test was used and statistically analysed at the 95% confidence level. *In vitro* data was analysed using a one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. *In vivo* data was analysed using a one-way analysis of variance (ANOVA) with a post-hoc Tukey's Multiple Comparison

test or a two-way ANOVA with post-hoc Bonferroni test and considered significant at a p value less than 0.05.

3. Results

3.1. The effect of salbutamol base, salbutamol sulphate and salbutamol in the presence of 1H2NA on lung function in vivo

An i.v. infusion of the peptide bombesin induced an increase in airways resistance in all animals (**Figure 1**). A 100 % increase in resistance was desired and the dose of bombesin was adjusted accordingly. Bombesin induced airway obstruction by 85 - 123 % in all animals.

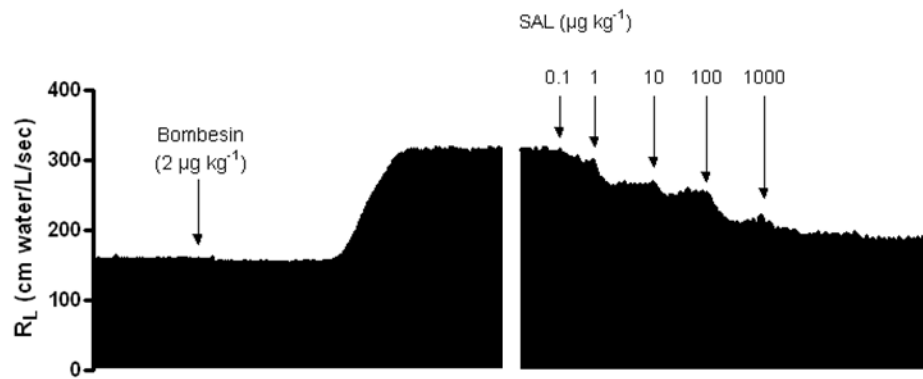


Figure 1. Representative trace of changes in total lung resistance following i.v. administration of bombesin ($2 \mu\text{g kg}^{-1}$), followed by i.v. administration of salbutamol base (SAL) at regular intervals (0.1, 1, 10, 100 and $1000 \mu\text{g kg}^{-1}$).

Salbutamol base (0.1, 1, 10, 100 and 1000 $\mu\text{g kg}^{-1}$), salbutamol sulphate (0.1, 1, 10, 100 and 1000 $\mu\text{g kg}^{-1}$) and salbutamol in the presence of 1H2NA (0.1:1, 1:10, 10:100 $\mu\text{g kg}^{-1}$) all elicited a dose-related reduction in the bombesin induced resistance (**Figure 2**). These data seem to suggest that the bronchodilator effect of the different salbutamol products differed between the treatment groups (**Figure 2, Supporting Information Figure S1**). Salbutamol in the presence of 1H2NA at 10 $\mu\text{g kg}^{-1}$ reversed peak resistance by $81.30 \pm 3.59\%$ ($n = 7$) ($p < 0.05$, two-way ANOVA with post-hoc Bonferroni test vs. salbutamol base), whereas at a similar dose, salbutamol sulphate only reversed peak resistance by $59.44 \pm 6.78\%$ ($n = 5$), and salbutamol base by only $52.47 \pm 11.92\%$.

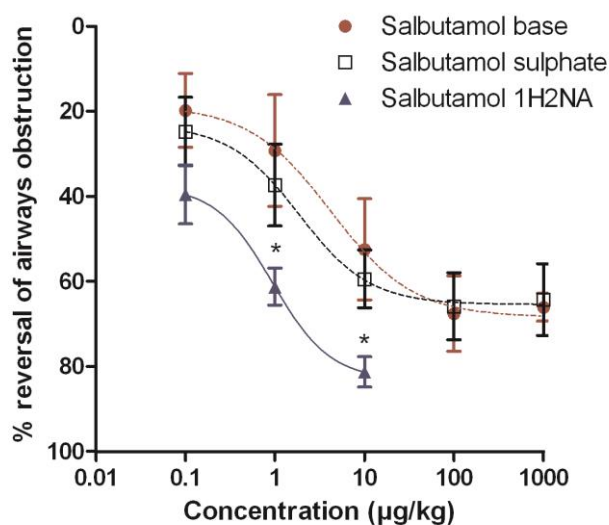


Figure 2. Bronchodilating effect of Salbutamol base (0.1, 1, 10, 100 and 1000 $\mu\text{g kg}^{-1}$, pH 7.4), salbutamol sulphate (0.1, 1, 10, 100 and 1000 $\mu\text{g kg}^{-1}$, pH 7.4) and salbutamol in the presence of 1H2NA (0.1:1, 1:10, 10:100 $\mu\text{g kg}^{-1}$, pH 7.4) on bombesin-induced increase in total lung resistance. Points represent mean \pm S.E.M; $n = 5 - 7$. Two-way ANOVA with post-hoc Bonferroni test, (*) $p < 0.05$ vs. salbutamol base.

3.2. The effect counter-ions on lung function upon administration of formoterol and salmeterol

Formoterol base (0.1, 1 and 10 $\mu\text{g kg}^{-1}$), formoterol fumarate (0.1:1, 1:10, 10:100 $\mu\text{g kg}^{-1}$), formoterol in the presence of maleate, and formoterol in the presence of aspartate (0.1:1, 1:10, 10:100 $\mu\text{g kg}^{-1}$) all elicited a dose-related reduction in resistance induced by bombesin (**Figure 3, Supporting Information Figure S2**). However, the percentage reversal of airway obstruction of the different formoterol products was very similar (**Figure 3**).

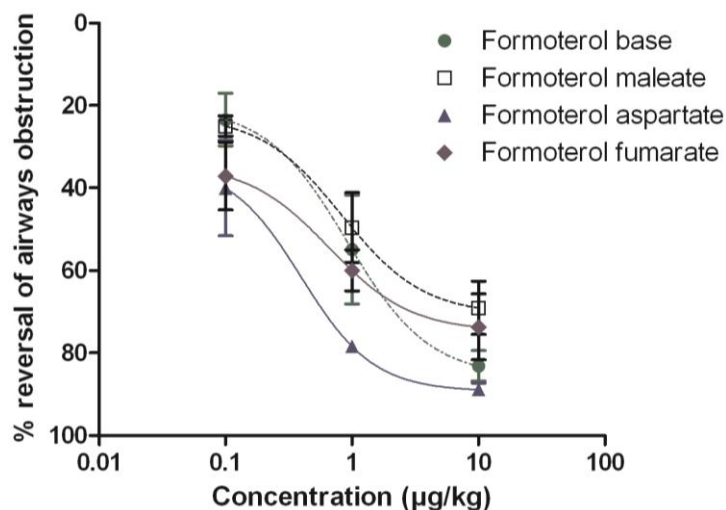


Figure 3. Bronchodilator effect of formoterol base (0.1, 1 and 10 $\mu\text{g kg}^{-1}$, pH 7.4), formoterol fumarate (0.1, 1 and 10 $\mu\text{g kg}^{-1}$, pH 7.4), formoterol in the presence of maleate, and formoterol in the presence of aspartate (0.1:1, 1:10 and 10:100 $\mu\text{g kg}^{-1}$, pH 7.4) on bombesin-induced increases in total lung resistance. Points represent mean \pm S.E.M; $n = 3 - 4$.

Salmeterol base appeared to have a very different dose-response profile compared to salbutamol and formoterol; for example, salmeterol displayed very little relaxation of the tissue at 0.1 and 1 $\mu\text{g kg}^{-1}$ unlike the other bronchodilators (**Figure 4, Supporting Information Figure S3**). However, as with formoterol, the presence of counter-ions did not seem to influence the performance of salmeterol, i.e., all the dose response profiles were similar both with and without the salt counter-ions (**Figure 4**). Only salmeterol maleate showed a significantly slower effect at reversing peak resistance compared to salmeterol 1H2NA, but only at 10 $\mu\text{g kg}^{-1}$ ($p < 0.05$, two-tailed unpaired, Student's t -test) (**Figure 4**).

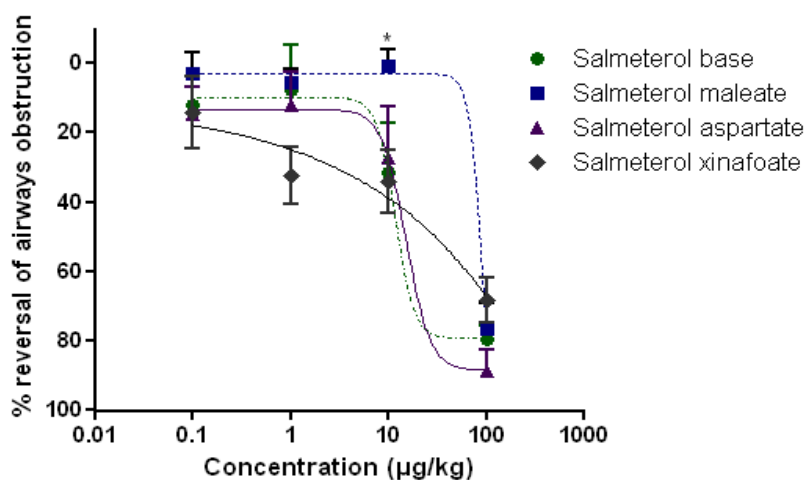


Figure 4. Bronchodilator effect of salmeterol base (0.1, 1, 10 and 100 $\mu\text{g kg}^{-1}$, pH 7.4), salmeterol xinafoate (0.1, 1, 10 and 100 $\mu\text{g kg}^{-1}$, pH 7.4), salmeterol in the presence of maleate and salmeterol in the presence of aspartate (0.1:1, 1:10, 10:100 and 100:1000 $\mu\text{g kg}^{-1}$, pH 7.4) on bombesin-induced increase in total lung resistance. Points represent mean \pm S.E.M; $n = 3 - 5$.

Two-tailed unpaired, Student's t -test. (*) $p < 0.05$ vs. salmeterol xinafoate.

3.3. Salbutamol counter-ion interaction studies

Using Fourier transform infrared spectroscopy (FT-IR) measurements the salbutamol base sample showed a peak at 1606 cm^{-1} in solution, which was assigned to the secondary amine (**Figure 5**). A significant shift in this secondary amine bend was observed for salbutamol base (1606 cm^{-1}) compared to salbutamol presented as the commercial sulphate (1617 cm^{-1}) (**Supporting Information Figure S4**). The N - H bend ($\sim 1616\text{ cm}^{-1}$) of salbutamol was also modified with increasing concentrations of sulphate (**Figure 5**). At 35 mM sulphate, a peak height ratio (1606 cm^{-1} and 1617 cm^{-1}) change was detected. The peak height ratio continued to change with increasing sulphate concentrations until the 1606 cm^{-1} peak disappeared with 158 mM sulphate. The peak height ratio, determined using the peaks at $\sim 1606\text{ cm}^{-1}$ and $\sim 1618\text{ cm}^{-1}$, was used to calculate the percentage of salbutamol bound to sulphate in solution and this was plotted against the free sulphate concentration in order to calculate a log affinity constant of 0.85 ± 0.03 , assuming a 1:1 association (note: we did not consider a 1:2, salbutamol sulphate ratio to be relevant in solution due to the weak binding and the relatively low amount of counter-ion present). In the presence of 1H2NA the spectral splitting of the 1615 peak indicated that an ion-pair was formed. However, these spectral changes occurred at a 10 fold lower concentration compared to the sulphate counter-ion. Unlike for the sulphate counter-ion the overlapping signals made it impossible to calculate the peak ratio and construct a binding curve (**Figure 5**). Attempts were made to use UV/Vis spectroscopy to support the IR data, but the salbutamol base λ_{max} at 276.43 nm did not shift (**Supporting Information Figure S5**). To facilitate the subsequent *in vitro* and *in vivo* studies, the HYSS microspeciation software (Protonic Software, UK) was used to calculate the optimum ratio for salbutamol: counter-ion that would generate ion-pairs at pH 7.4 employing the measured affinity constant for salbutamol sulphate (given that

the 1H2NA binding was ca. 10-fold stronger). These results suggested that at a 1:10 drug to counter-ion molar ratio with the ion-paired species would be formed (data not shown).

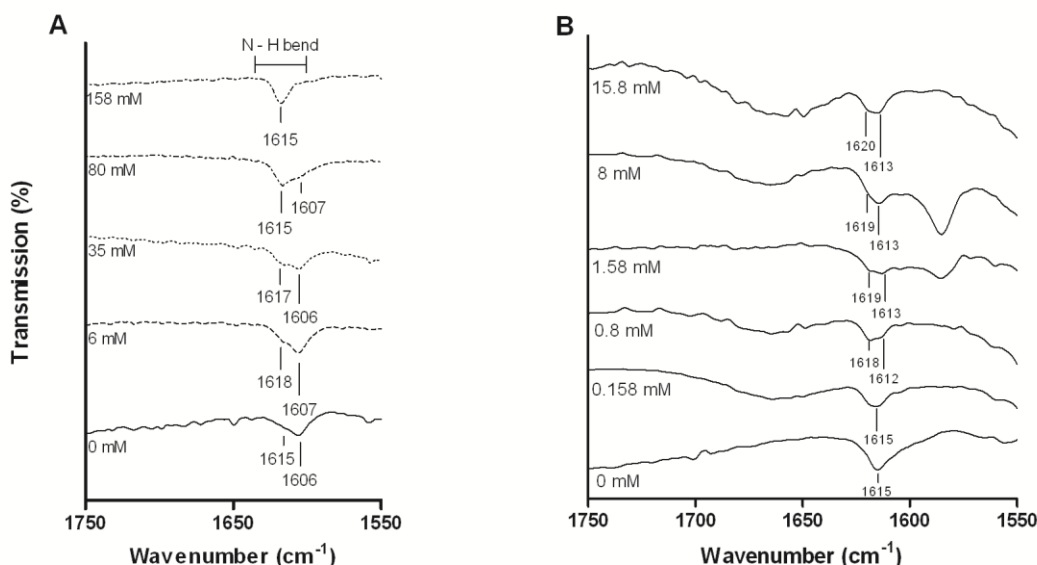


Figure 5. FT-IR spectra of (A) salbutamol base and salbutamol in the presence of increasing sulphate and (B) salbutamol base in the presence of increasing 1H2NA concentrations in the liquid state (80 % deuterated water and 20 % deuterated methanol, pH 6.5). The number denotes the concentration (mM) of counter-ion present against a fixed salbutamol base concentration (42 mM).

3.4. Salbutamol transport across biological membranes

The flux of the commercial salbutamol sulphate mixture through the tracheal tissue was $1.17 \pm 0.27 \mu\text{g}/\text{cm}^2/\text{min}$ (**Figure 6**). This flux for salbutamol sulphate was similar to salbutamol 1H2NA ($1.49 \pm 0.14 \mu\text{g}/\text{cm}^2/\text{min}$), but both systems containing the counter-ions displayed a significantly greater flux than that of salbutamol base alone at $0.34 \pm 0.07 \mu\text{g}/\text{cm}^2/\text{min}$ ($p <$

0.0001, two-tailed, unpaired, Student's *t*-test). The cumulative mass of salbutamol base (0.00178 M) that transported into the receiver solution was $97.30 \pm 18.94 \mu\text{g}/\text{cm}^2$ at 360 min (**Figure 6**). The cumulative mass of salbutamol (0.00178 M) that transported across the guinea pig tracheal tissue when presented with an excess of the hydrophobic counter-ion, 1H2NA (0.0178 M) at 360 min was $335.08 \pm 28.16 \mu\text{g}/\text{cm}^2$ and this was significantly greater ($p < 0.0001$, two-tailed, unpaired, Student's *t*-test) than salbutamol base alone (**Figure 6**). The lag time to steady-state flux of salbutamol in the presence of 1H2NA was significantly higher than that of the base (138.91 ± 8.27 min with 1H2NA vs. 87.60 ± 5.54 min for the base; $p < 0.0001$, two-tailed, unpaired, Student's *t*-test), whereas the lag time for salbutamol sulphate crossing the lung tracheal tissue was lower than the base at 28.97 ± 6.52 min ($p < 0.0001$, two-tailed, unpaired, Student's *t*-test). The apparent permeability (P_{app} (cm/s)) of salbutamol base in the presence of 1H2NA and in the presence of sulphate was also significantly greater than the base alone ($p < 0.0001$, two-tailed, unpaired, Student's *t*-test) (**Table 1**).

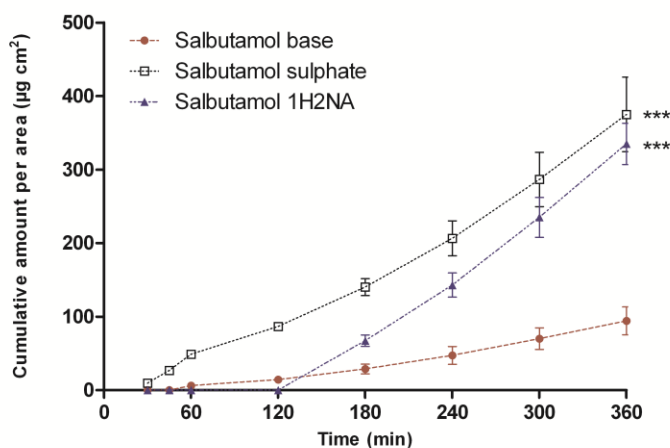


Figure 6. Transport of salbutamol base (0.00178 M, pH 7.4), salbutamol sulphate (0.00178 M : 0.00037 M, pH 7.4) and salbutamol in the presence of excess 1H2NA (0.00178 : 0.0178 M, pH 7.4) across guinea pig lung tracheal tissue into saline (pH 7.4). Each point represents the mean cumulative mass per area \pm SD ($n = 5-12$). Two-tailed, unpaired, Student's *t*-test, (***) $p < 0.0001$ vs. salbutamol base.

Table 1. The flux ($\mu\text{g}/\text{cm}^2/\text{min}$) and apparent permeability (P_{app}) of salbutamol base, salbutamol in the presence of sulphate, and salbutamol in the presence of excess 1H2NA, through guinea pig tracheal tissue into saline pH 7.4 at 37 °C. The concentration of salbutamol remained constant at 0.00178 M. Each value of the flux represents the mean \pm standard deviation.

Drug	Detection (min)	Slope vs. Time (min)	Flux ($\mu\text{g}/\text{cm}^2/\text{min}$)	R^2	P_{app} (cm/s)	n
Salbutamol base	60	120 - 360	0.34 ± 0.07	0.9992	$1.31 \pm 0.28 \times 10^{-5}$	5
Salbutamol Sulphate (commercial)	30	60 - 360	1.17 ± 0.27	0.9717	$4.57 \pm 1.08 \times 10^{-5}$	12
Salbutamol 1H2NA	180	180 - 360	1.49 ± 0.14	0.9964	$5.84 \pm 0.57 \times 10^{-5}$	5

3.5. The inhibitory actions of salbutamol, salbutamol sulphate and salbutamol in the presence of 1H2NA on EFS-induced contractions of isolated guinea pig tracheal smooth muscle

The superfusion of salbutamol base (1 μM) in de-ionised water (pH 7.4) caused an 84.24 ± 0.64 % inhibition of the tissues with an OT_{50} of 5.00 ± 0.00 min and an OT_{75} of 8.75 ± 1.03 min. Both the OT_{50} and OT_{75} for salbutamol sulphate was significantly faster compared to salbutamol base alone ($p < 0.01$ and $p < 0.05$ respectively, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test) (**Table 2**). However, the total percentage inhibition of the tissue superfused with salbutamol sulphate was similar to the base ($p > 0.05$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test). The tissue recovery indices of the base and sulphate forms of salbutamol were also similar ($p > 0.05$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test). All of the tissues recovered to 100% of the pre-drug value and the tissues that had been treated with salbutamol were not

significantly different ($p > 0.05$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test) compared to the drug free controls (**Table 2**).

Salbutamol (1 μM) in the presence of excess 1H2NA (10 μM) had an OT_{50} similar to that of salbutamol base ($p > 0.05$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test). However, the OT_{75} for salbutamol in the presence of 1H2NA was significantly faster with an onset time of 4.38 ± 0.89 minutes compared to 8.75 ± 1.03 minutes for the base ($p < 0.05$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test). The inhibition of contraction by salbutamol in the presence of 1H2NA was also greater than salbutamol base alone, with a total inhibition of 94.13 % ($p < 0.05$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test). The time for the tissues to reach 50 % recovery compared to the pre-drug values, for both salbutamol base and salbutamol in the presence of 1H2NA, was approximately 50 minutes ($p > 0.05$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test). However, the average time taken for the tissues to recover to 75% of the pre-drug value for salbutamol in the presence of 1H2NA was faster ($p < 0.01$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test). All of the tissues recovered to 100 % of the pre-drug value and were not significantly different from both controls ($p > 0.05$, one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test) (**Table 2**).

Table 2. The percentage control (%) of tissues after drug addition, and percentage tissue recovery at 360 minutes post dosing, onset time (OT_{50} , OT_{75}) to achieve 50% or 75% control (pre-drug) value and recovery time (RT_{50} , RT_{75}) to return to 50% or 75% of control (pre-drug)

value (minutes) for salbutamol base (SAL, 1 μ M), salbutamol sulphate (SAL Sulphate, 1 μ M) and salbutamol in the presence of 1H2NA (1 μ M: 10 μ M) at pH 7.4 in isolated guinea pig trachea. Results expressed as mean \pm SEM. A one way ANOVA Kruskal-Wallis test with post-hoc Dunn's multiple comparison test was used for statistical comparison: (*) $p < 0.05$ vs. salbutamol base; (**) $p < 0.01$ vs. salbutamol base.

Test substance					
	Control	Water	SAL	SAL Sulphate	SAL + 1H2NA
OT₅₀ (min)	---	---	5.00 \pm 0.00	2.51 \pm 0.00**	4.37 \pm 0.62
OT₇₅ (min)	---	---	8.75 \pm 1.03	3.89 \pm 0.97*	4.38 \pm 0.84*
Control (%)	108.19 \pm 5.90	106.04 \pm 4.97	15.79 \pm 0.64	17.98 \pm 2.33	5.87 \pm 1.96**
RT₅₀ (min)	---	---	53.34 \pm 2.64	53.34 \pm 0.00	51.25 \pm 2.08
RT₇₅ (min)	---	---	84.17 \pm 2.50	74.17 \pm 0.00	61.67 \pm 0.00**
Control (360 min; %)	108.19 \pm 3.74	104.88 \pm 4.89	101.97 \pm 2.13	99.07 \pm 2.11	102.64 \pm 2.98
N	5	3	5	3	4

4. Discussion

Of the eight different drug-counter-ion combinations assessed *in vitro*, only the salbutamol 1H2NA complex had an effect on airway smooth muscle (ASM) relaxation when compared with salbutamol base. It could be argued that the enhanced smooth muscle relaxation in the presence of 1H2NA could have been a consequence of this molecule having an independent action on ASM as it was presented to the tissue in a molar excess compared to salbutamol base. There is little information in the literature regarding the effect of 1H2NA on biological tissues, but Groeben and Emala¹⁹ did evaluate the effect of xinafoic acid (1H2NA) on ASM, in particular its ability to bind to the β_2 -AR. In this previous work it was concluded that 1H2NA had no direct airway irritant effects, had also no direct bronchodilating effects *in vivo*

and it was thought that it did not interfere with β_2 -AR binding *in vitro*, nor impair the binding of salmeterol to β_2 -AR. The data from the present study, which showed an effect of 1H2NA with salbutamol, but not with salmeterol, concurred with the previous suggestions that the 1H2NA effects were not dependent on any direct interaction with β_2 -receptors.

The ability of drug-ion pair formation to enhance drug absorption has been reported in the literature.^{1,6,20} In addition, it has already been established in previously published work that a number of anionic drugs can form complexes with 1H2NA. However, there seems to be an absence of studies that determine the ion-pair association using direct spectroscopy and then go on to understand their pharmacological effects. This was presumably because the ionic and other non-covalent interactions (i.e. hydrogen bonding) that occur between the ionic drug and the counter-ion during ion-pair formation are relatively weak,^{21,22} and in aqueous solvents the water signals make the detection of any spectra shifts difficult to observe.^{23,24} Despite these issues, in the present study, we attempted to generate direct spectroscopy evidence of ion-pair formation, but as a simple aqueous solution was not capable of dissolving salbutamol and the test counter-ions, methanol was added to aid solubility. It was known that the presence of methanol would influence the absolute values for the affinity constant compared to a pure water system, but it was believed that by keeping a constant solvent system across the measurements it would not detract from the comparative measurements made in the current study. In addition, there are methods available to predict affinity constants in water from experimental data with water methanol solvents.²⁵

The FT-IR spectroscopy data generated in this study substantiated that salbutamol formed an ion-pair with sulphate and 1H2NA. However, a full binding curve could only be obtained for the salbutamol sulphate salt. The spectral shifts for 1H2NA occurred at a 10 fold lower

concentration compared to salbutamol, and despite not being able to generate an absolute value for the binding affinity between salbutamol-1H₂NA, the data allowed us to predict with some confidence that the salbutamol-1H₂NA ion-pair would be formed at the 1:10 ratio of drug:counter-ion that was used across all the experiments.²⁶

For salbutamol, which is thought to enter the tissue primarily via the paracellular route,²⁷ the formation of the ion-pairs could have modified tissue uptake by making the transcellular route more favourable since the formation of a complex would have to some extent reduced the charge and hydrophilicity of the salbutamol molecule.¹ The comparative effects of this counter-ion enhanced drug transport process would be much less for formoterol and salmeterol because it is thought that these molecules are naturally transported by the transcellular route, even without the addition of a counter-ion, due to their greater hydrophobicity.²⁸ It has been hypothesised that once deposited into the airways formoterol moves efficiently across the airway epithelium and into the lamina propria where it diffuses towards ASM by partitioning into the lipid bilayer and then progressively leaches out to interact with the β_2 -AR active site.²⁹ Salmeterol, contains a longer side chain than the shorter acting β_2 -agonist salbutamol that makes it > 10 000 times more lipophilic and so it has also been hypothesised to move readily through the epithelial cell membrane.³⁰

Although superfusion studies were not completed in this work for salmeterol or formoterol, previous studies have shown that both formoterol and salbutamol display a faster onset time compared to salmeterol.^{31,32} Furthermore, formoterol is known to be more potent than salmeterol in both *in vitro* and *in vivo* studies.^{31,32} This information from the literature compliments the lung function studies in the current work where formoterol seemed to have a greater degree of inhibition compared to salmeterol alone and in the presence of counter-ions

across the concentration range ($0.1 - 10 \mu\text{g kg}^{-1}$).³³ This suggested that the *in vivo* systems used in this study were working well. The lack of a significant difference observed when the counter-ions were combined with formoterol (figure 3) or salmeterol (figure 4) may be due to the choice of counter-ions. The ability to form an ion-pair that will remain complexed for long enough to influence its pharmacological behaviour, is related to the chemical structure and physicochemical properties of the parent drug and the counter-ions present. Although, no clear significant differences were observed the curves for formoterol and salmeterol in the presence of counter maleate was significantly different from salmeterol xinafoate, and the dose response profiles for salmeterol base and salmeterol aspartate were very different to salmetrol xinafoate (figure 4). These small, but significant differences could be exploited by investigating alternative counter ions to translate into further significant biological effects if the alternative counter-ions used had a stronger affinity for the parent drug.

As the 1H2NA counter-ion has been previously shown to form ion-pairs with a wide range of drug molecules,¹ it was thought that it would be worthwhile to further understand the transport of salbutamol 1H2NA into lung tissue using an *in vitro* model. As the influence of ion-pairing on a range of cell lines has previously been established *in vitro*,^{1,34} in this work we wanted to use ex-vivo tissue such that we could understand not only passage through the epithelial barrier, but also drug permeation across through pulmonary tissue as this has relevance to both locally administered and systemically administered agents. Although we accept only a limited amount of drug is absorbed through the tracheal tissue, this section of the respiratory tract provided significant rigidity to allow drug transport to be assessed. A 60 min equilibration time was needed to ensure the Franz cells were secure, free of bubbles and at a temperature of 37°C .^{35,36} As these conditions remained constant throughout the work it was unlikely that the

application of the liquid, which can change apical ion channel abundance, and hence cause increased fluxes due to activation of fluid homoeostasis mechanisms had an effect on the data set trends.³⁷

For the *in vitro* lung trachea transport studies the hypothesis was that ion-pair formation enhanced the ability of salbutamol to move into the tissue. Salbutamol base at pH 7.4 is very hydrophilic with a cLog $D_{7.4}$ of -1.32 (MarvinSketch, ChenAxon Ltd) and it is fully ionised (calculated amine $pK_a = 9.02 \pm 0.028$) from pH 8 and below; therefore there is no pH dependant change in the transport of salbutamol across this range.³⁸ Although, lung lining fluid is slightly acidic (pH 6.9)³⁹ the transport studies were conducted at pH 7.4 to prevent a pH gradient across the cells as this could influence transport. Furthermore, a pH of 7.4 is typically used in cell culture models when characterising salbutamol transport.⁴⁰ These physicochemical properties enable fast dissolution of the parent drug to occur when it is administered as a solid, but salbutamol's polarity results in only a moderate affinity with the cellular epithelium of tracheal tissue. Evidence for the transcellular route being the main path of entry into the pulmonary tissue has been generated through transport inhibition studies using other charged molecules such as amino acids.²⁷ More recent studies have also suggested the involvement of organic cation transporters (OCTs) in tissue accumulation and cellular uptake of inhaled drugs.^{41,42} As salbutamol base carries a net positive charge at physiological lung pH,⁴³ OCTs may be involved in the absorption and clearance processes for this drug in the lung without the formation of a ion-pair,^{42,43} but the OCT's capacity to take up a neutral ion-pair would presumably be diminished.

The association of 1H2NA with salbutamol would generate a larger, more neutral molecule which would be inherently more hydrophobic than the parent.^{34,44} It is these changes in physicochemical properties which would most likely drive the increase in affinity of the ion-pair

for the lung tissue compared to the parent drug.^{45,46} The increase in transport lag-time, which traditionally occurs as a consequence of an increase in molecular size of a compound,⁴⁷ and the higher P_{app} of salbutamol in the presence of 1H2NA support this hypothesis. ~~Salbutamol P_{app} across Calu-3 monolayer's was determined to be $1.99 \pm 0.25 \times 10^{-6}$ cm/s⁴³ which was only 10 fold slower when compared to lung tracheal tissue.~~ The P_{app} values reported in the literature for salbutamol range by 2 orders of magnitude from 1.5×10^{-8} (porcine tissue)⁴⁸ to 5.2×10^{-6} cm/s (Calu-3 cells).⁴⁹ The salbutamol base P_{app} of $1.31 \pm 0.28 \times 10^{-5}$ cm/s reported in the current work was approximately double the highest of these previously reported values and this was considered to be due to the different permeability of guinea pig trachea. No previous work has used this tissue to test drug transport and the transport data suggested that the barrier was confluent.

The Sulphate counter-ion could also have a similar effect to 1H2NA on salbutamol transport, but the charging on the sulphate at pH 7.4 would mean that a neutral complex is unlikely to be formed and the transport of the salbutamol sulphate may be a little more complex than the 1H2N salbutamol ion-pair. However, it was noteworthy that the sulphate counter-ion displayed the ability to alter the transport into the tissue, even at the 1:2 drug:counter-ion ratio. The cLog $D_{7.4}$ of 1H2NA is predicted to be -0.53 (MarvinSketch software (ChemAxon)) making it a more lipophilic ion-pairing agent compared to the sulphate, cLog $D_{7.4}$ -5.54 (MarvinSketch software (ChemAxon)). Assuming that salbutamol and 1H2NA are forming an ion-pair in solution, the overall complex would be more hydrophobic compared to the sulphate counter-ion complex and thus one may expect the former to show greater affinity to tissue and a greater propensity to be transported via the transcellular route.⁴⁶ Ion-pair transport across the membrane

will not be hindered by charged proteins in the ECM as may be the case for weak bases such as salbutamol⁵⁰ and this could aid the transport rates across the tissue.

The *in vitro* studies that investigated the relaxant activity of salbutamol in the presence of the counter-ions in isolated guinea pig tracheal tissue showed only a marginal effect of the salbutamol:1H₂NA complex. The small magnitude of the response in the isolated tissue model suggested that the pharmacology of the drugs action was not dramatically modified as a consequence of ion-pair formation. Therefore, it was proposed that the ion-pair broke down once taken up into the tissue. This was supported by the fact that the weaker of the two ion-pairs, the salbutamol sulphate complex, did not show any effect. It was interesting however that the delayed lag time showed in the transport studies did not have any consequences on the pharmacological indices measured both *in vitro* and *in vivo*. In fact the 1H₂NA ion-pair had a significantly faster onset rate (OT₇₅ only) in addition to a greater degree of inhibition compared to the base alone, which would not be predicted when analysing drug transport across lung trachea. It was also surprising that the faster on-set of pharmacological action was coupled with a significantly faster recovery rate, not observed with salbutamol sulphate. It was expected that the 1H₂NA counter-ion would provide a slower recovery rate due to an increase in the hydrophobicity of the complex, but perhaps if the ion-pair complex was dissociating in the tissue this resulted in a complex relationship between the transport and pharmacological activity.

5. Conclusions

The results from this study provide evidence that the counter-ions included in pharmaceutical salts are important in determining the biological behaviour of the parent drug

molecule when delivered by the inhaled route. These results need confirming using pharmaceutical salts in the solid state in man, but if this is achieved it will have significant implications for the choice of counter-ion for inhaled drugs. These data from our study suggest that when counter-ions are switched in generic products, careful characterisation of the pharmaceutical alternative's properties must be undertaken prior to medicine approval.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website. Bronchodilating effect of salbutamol, formoterol and salmeterol, FT-IR spectra of salbutamol base and salbutamol sulphate and ultraviolet scan of salbutamol base in the presence of increasing 1H₂NA concentrations.

AUTHOR INFORMATION

Corresponding Author

E-mail: aateka.patel@kcl.ac.uk; Tel: +44 207 848 4819

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

The authors would like to thank MedPharm and the EPSRC (Industrial CASE number 09001807) for funding this study.

REFERENCES

- (1) Miller, J. M.; Dahan, A.; Gupta, D.; Varghese, S.; Amidon, G. L. Quasi-Equilibrium Analysis of the Ion-Pair Mediated Membrane Transport of Low-Permeability Drugs. *J. Control. Release* **2009**, *137* (1), 31–37.
- (2) Stahl, P. H.; Wermuth, C. G. *Handbook of Pharmaceutical Salts Properties, Selection, and Use*; Stahl, P. H., Wermuth, C. G., Eds.; Wiley-VCH, Weinheim, 2002.
- (3) Neubert, R. Ion Pair Transport across Membranes. *Pharmaceutical research*. 1989, pp 743–747.
- (4) Patel, A.; Jones, S. A.; Ferro, A.; Patel, N. Pharmaceutical Salts: A Formulation Trick or a Clinical Conundrum? *Br. J. Cardiol.* **2009**, *16* (6), 281–286.
- (5) Quintanar-Guerrero, D.; Allémann, E.; Fessi, H.; Doelker, E. Applications of the Ion-Pair Concept to Hydrophilic Substances with Special Emphasis on Peptides. *Pharmaceutical Research*. 1997, pp 119–127.
- (6) Irwin, G. M.; Kostenbauder, H. B.; Dittert, L. W.; Staples, R.; Misher, A.; Swintosky, J. V. Enhancement of Gastrointestinal Absorption of a Quaternary Ammonium Compound by Trichloroacetate. *J. Pharm. Sci.* **1969**, *58* (3), 313–315.
- (7) Tan, Z.; Zhang, J.; Wu, J.; Fang, L.; He, Z. The Enhancing Effect of Ion-Pairing on the Skin Permeation of Glipizide. *AAPS PharmSciTech* **2009**, *10* (3), 967–976.
- (8) Tantishaiyakul, V.; Phadoongsombut, N.; Wongpuwarak, W.; Thungtiwachgul, J.; Faroongsarng, D.; Wiwattanawongsa, K.; Rojanasakul, Y. ATR-FTIR Characterization of Transport Properties of Benzoic Acid Ion-Pairs in Silicone Membranes. *Int. J. Pharm.* **2004**, *283* (1-2), 111–116.
- (9) Fang, L.; Xi, H.; Cun, D. Formation of Ion Pairs and Complex Coacervates. In *Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement*; Springer Berlin Heidelberg: Berlin, Heidelberg, 2015; pp 175–187.
- (10) Jashnani, R. N.; Dalby, R. N.; Byron, P. R. Preparation, Characterization, and Dissolution Kinetics of Two Novel Albuterol Salts. *J. Pharm. Sci.* **1993**, *82* (6), 613–616.
- (11) Montuschi, P.; Ciabattoni, G. Bronchodilating Drugs for Chronic Obstructive Pulmonary Disease: Current Status and Future Trends. *Journal of Medicinal Chemistry*. 2015, pp 4131–4164.

- (12) Paluch, K. J.; Tajber, L.; Elcoate, C. J.; Corrigan, O. I.; Lawrence, S. E.; Healy, A. M. Solid-State Characterization of Novel Active Pharmaceutical Ingredients: Cocrystal of a Salbutamol Hemiadipate Salt with Adipic Acid (2:1:1) and Salbutamol Hemisuccinate Salt. *J. Pharm. Sci.* **2011**, *100* (8), 3268–3283.
- (13) Brittain, R. T.; Farmer, J. B.; Marshall, R. J. Some Observations on the β -Adrenoceptor Agonist Properties of the Isomers of Salbutamol. *Br. J. Pharmacol.* **1973**, *48* (1), 144–147.
- (14) Handley, D. a; Anderson, a J.; Koester, J.; Snider, M. E. New Millennium Bronchodilators for Asthma: Single-Isomer Beta Agonists. *Curr. Opin. Pulm. Med.* **2000**, *6* (1), 43–49.
- (15) Jashnani, R. N.; Byron, P. R. Dry Powder Aerosol Generation in Different Environments: Performance Comparisons of Albuterol, Albuterol Sulfate, Albuterol Adipate and Albuterol Stearate. *Int. J. Pharm.* **1996**, *130* (1), 13–24.
- (16) Barnes, P. J.; Grandordy, B. M.; Page, C. P.; Rhoden, K. J.; Robertson, D. N. The Effect of Platelet Activating Factor on Pulmonary Beta-Adrenoceptors. *Br. J. Pharmacol.* **1987**, *90* (4), 709–715.
- (17) Coleman, R. a; Nials, a T. Novel and Versatile Superfusion System. Its Use in the Evaluation of Some Spasmogenic and Spasmolytic Agents Using Guinea-Pig Isolated Tracheal Smooth Muscle. *J. Pharmacol. Methods* **1989**, *21* (1), 71–86.
- (18) Boswell-smith, V.; Spina, D.; Oxford, A. W.; Comer, M. B.; Seeds, E. a; Page, C. P. The Pharmacology of Two Novel Long-Acting. *Pharmacology* **2006**, *318* (2), 840–848.
- (19) Groeben, H.; Emala, C. Is Beta-Adrenergic-Mediated Airway Relaxation of Salmeterol Antagonized by Its Solvent Xinafoic Acid? *Chest* **1999**, *115* (6), 1678–1683.
- (20) Jonkman, J. H.; Hunt, C. a. Ion Pair Absorption of Ionized Drugs--Fact or Fiction? *Pharm. Weekbl. Sci.* **1983**, *5* (2), 41–48.
- (21) Dai, J.; Carr, P. W. Role of Ion Pairing in Anionic Additive Effects on the Separation of Cationic Drugs in Reversed-Phase Liquid Chromatography. *J. Chromatogr. A* **2005**, *1072* (2), 169–184.
- (22) Williams, H. D.; Trevaskis, N. L.; Charman, S. a; Shanker, R. M.; Charman, W. N.; Pouton, C. W.; Porter, C. J. H. Strategies to Address Low Drug Solubility in Discovery and Development. *Pharmacol Rev* **2013**, *65* (1), 315–499.
- (23) Hefter, G. When Spectroscopy Fails: The Measurement of Ion Pairing. *Pure Appl. Chem.* **2006**, *78* (8).

- (24) Burba, C. M.; Janzen, J.; Butson, E. D.; Coltrain, G. L. Using FT-IR Spectroscopy to Measure Charge Organization in Ionic Liquids. *J. Phys. Chem. B* **2013**, *117* (29), 8814–8820.
- (25) Jouyban, A.; Chan, H.; Clark, B. J.; Acree, W. E. Mathematical Representation of Apparent Dissociation Constants in Aqueous–organic Solvent Mixtures. *Int. J. Pharm.* **2002**, *246* (1-2), 135–142.
- (26) Valenta, C.; Siman, U.; Kratzel, M.; Hadgraft, J. The Dermal Delivery of Lignocaine: Influence of Ion Pairing. *Int. J. Pharm.* **2000**, *197* (1-2), 77–85.
- (27) Unwalla, H. J.; Horvath, G.; Roth, F. D.; Conner, G. E.; Salathe, M. Albuterol Modulates Its Own Transepithelial Flux via Changes in Paracellular Permeability. *Am. J. Respir. Cell Mol. Biol.* **2012**, *46* (4), 551–558.
- (28) Anderson, G. P. Formoterol: Pharmacology, Molecular Basis of Agonism, and Mechanism of Long Duration of a Highly Potent and Selective β_2 -Adrenoceptor Agonist Bronchodilator. *Life Sci.* **1993**, *52* (26), 2145–2160.
- (29) Johnson, M. Beta2-Adrenoceptors: Mechanisms of Action of beta2-Agonists. *Paediatr. Respir. Rev.* **2001**, *2* (1), 57–62.
- (30) Johnson, M.; Butchers, P. R.; Coleman, R. a; Nials, a T.; Strong, P.; Sumner, M. J.; Vardey, C. J.; Whelan, C. J. The Pharmacology of Salmeterol. *Life Sci.* **1993**, *52* (26), 2131–2143.
- (31) Lindén, a; Bergendal, A.; Ullman, A.; Skoogh, B. E.; Löfdahl, C. G. Salmeterol, Formoterol, and Salbutamol in the Isolated Guinea Pig Trachea: Differences in Maximum Relaxant Effect and Potency but Not in Functional Antagonism. *Thorax* **1993**, *48* (5), 547–553.
- (32) Battram, C.; Charlton, S. J.; Cuenoud, B.; Dowling, M. R.; Fairhurst, R. a; Farr, D.; Fozard, J. R.; Leighton-Davies, J. R.; Lewis, C. a; McEvoy, L.; Turner, R. J.; Trifilieff, A. In Vitro and in Vivo Pharmacological Characterization of 5-[(R)-2-(5,6-Diethyl-Indan-2-Ylamino)-1-Hydroxy-Ethyl]-8-Hydroxy-1H-Quinolin-2-One (indacaterol), a Novel Inhaled beta(2) Adrenoceptor Agonist with a 24-H Duration of Action. *J. Pharmacol. Exp. Ther.* **2006**, *317* (2), 762–770.
- (33) Anderson, G. P.; Lindén, A.; Rabe, K. F. Why Are Long-Acting Beta-Adrenoceptor Agonists Long-Acting? *Eur. Respir. J.* **1994**, *7* (3), 569–578.
- (34) Miller, J. M.; Dahan, A.; Gupta, D.; Varghese, S.; Amidon, G. L. Enabling the Intestinal Absorption of Highly Polar Antiviral Agents: Ion-Pair Facilitated Membrane Permeation of Zanamivir Heptyl Ester and Guanidino Oseltamivir. *Mol. Pharm.* **2010**, *7* (4), 1223–1234.

- (35) Fitzpatrick, D.; Corish, J. Release Characteristics of Anionic Drug Compounds from Liquid Crystalline Gels. *Int. J. Pharm.* **2005**, *301* (1-2), 226–236.
- (36) Fiala, S.; Brown, M. B.; Jones, S. a. An Investigation into the Influence of Binary Drug Solutions upon Diffusion and Partition Processes in Model Membranes. *J. Pharm. Pharmacol.* **2008**, *60* (12), 1615–1623.
- (37) Tan, C. D.; Selvanathar, I. a.; Baines, D. L. Cleavage of Endogenous γ ENaC and Elevated Abundance of α ENaC Are Associated with Increased Na⁺ Transport in Response to Apical Fluid Volume Expansion in Human H441 Airway Epithelial Cells. *Pflügers Arch. - Eur. J. Physiol.* **2011**, *462* (3), 431–441.
- (38) Forbes, I. I. Human Airway Epithelial Cell Lines for in Vitro Drug Transport and Metabolism Studies. *Pharm Sci Technolo Today* **2000**, *3* (1), 18–27.
- (39) Patton, J. S. Mechanisms of Macromolecule Absorption by the Lungs. *Adv. Drug Deliv. Rev.* **1996**, *19* (1), 3–36.
- (40) Forbes, B.; Ehrhardt, C. Human Respiratory Epithelial Cell Culture for Drug Delivery Applications. In *European Journal of Pharmaceutics and Biopharmaceutics*; 2005; Vol. 60, pp 193–205.
- (41) Bäckström, E.; Lundqvist, A.; Boger, E.; Svanberg, P.; Ewing, P.; Hammarlund-Udenaes, M.; Fridén, M. Development of a Novel Lung Slice Methodology for Profiling of Inhaled Compounds. *J. Pharm. Sci.* **2015**, 1–9.
- (42) Salomon, J. J.; Hagos, Y.; Petzke, S.; Kühne, A.; Gausterer, J. C.; Hosoya, K.; Ehrhardt, C. Beta-2 Adrenergic Agonists Are Substrates and Inhibitors of Human Organic Cation Transporter 1. *Mol. Pharm.* **2015**, *12* (8), 2633–2641.
- (43) Ehrhardt, C.; Kneuer, C.; Bies, C.; Lehr, C.-M.; Kim, K.-J.; Bakowsky, U. Salbutamol Is Actively Absorbed across Human Bronchial Epithelial Cell Layers. *Pulm. Pharmacol. Ther.* **2005**, *18* (3), 165–170.
- (44) Miller, J. M. The Impact of Molecular Complexation on Intestinal Membrane Permeation, The University of Michigan, 2009.
- (45) Megwa, S. a; Cross, S. E.; Benson, H. a; Roberts, M. S. Ion-Pair Formation as a Strategy to Enhance Topical Delivery of Salicylic Acid. *J. Pharm. Pharmacol.* **2000**, *52*, 919–928.
- (46) Megwa, S. A.; Cross, S. E.; Whitehouse, M. W.; Benson, H. A.; Roberts, M. S. Effect of Ion Pairing with Alkylamines on the in-Vitro Dermal Penetration and Local Tissue Disposition of Salicylates. *J. Pharm. Pharmacol.* **2000**, *52* (8), 929–940.
- (47) Inacio, R. An Investigation into the Influence of Local Barometric Stress upon Xenobiotic Percutaneous Penetration, King's College London, 2016.

- (48) Van Zyl, J. M.; Derendinger, B.; Seifart, H. I.; Van der Bijl, P. Comparative Diffusion of Drugs through Bronchial Tissue. *Int. J. Pharm.* **2008**, 357 (1-2), 32–36.
- (49) Haghi, M.; Traini, D.; Bebawy, M.; Young, P. M. Deposition, Diffusion and Transport Mechanism of Dry Powder Microparticulate Salbutamol, at the Respiratory Epithelia. *Mol. Pharm.* **2012**, 9 (6), 1717–1726.
- (50) MacIntyre, a C.; Cutler, D. J. The Potential Role of Lysosomes in Tissue Distribution of Weak Bases. *Biopharm. Drug Dispos.* **2006**, 9 (6), 513–526.